The Endoplasmic Reticulum-Associated Degradation Pathways of Budding Yeast

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Protein misfolding is a common cellular event that can produce intrinsically harmful products. To reduce the risk, quality control mechanisms are deployed to detect and eliminate misfolded, aggregated, and unassembled proteins. In the secretory pathway, it is mainly the endoplasmic reticulum-associated degradation (ERAD) pathways that perform this role. Here, specialized factors are organized to monitor and process the folded states of nascent polypeptides. Despite the complex structures, topologies, and posttranslational modifications of client molecules, the ER mechanisms are the best understood among all protein quality-control systems. This is the result of convergent and sometimes serendipitous discoveries by researchers from diverse fields. Although major advances in ER quality control and ERAD came from all model organisms, this review will focus on the discoveries culminating from the simple budding yeast.

Secreted and membrane proteins insert into the endoplasmic reticulum (ER) through the Sec61 translocon complex as unfolded molecules (Rapoport 2007). In the lumen, protein folding is assisted by chaperones and modifying enzymes, some of which are members of ER quality control (ERQC) pathways. Chaperone interactions keep immature proteins soluble to prevent aggregation and facilitate folding. Once folding and assembly are complete, proteins are sorted for transport to their final destinations. Proteins failing to fold correctly are retained and targeted for degradation by the ER-associated protein degradation (ERAD) pathways of ERQC (McCracken et al. 1996). This

review will focus on mechanisms that differentiate folding intermediates, native proteins, and misfolded proteins in the ER and keep them on the correct processing track.

The discovery of protein quality control mechanisms in the ER originated from mammalian virus studies. The hemagglutinin (HA) and G glycoproteins of influenza and vesicular stomatitis viruses, respectively, fold and oligomerize in the ER (Copeland et al. 1986; Gething et al. 1986; Doms et al. 1988). Because these viruses bud from the plasma membrane, researchers observed that unfolded and misfolded molecules were stringently retained in the ER (Gething et al. 1986; Copeland et al. 1988; Doms et al. 1988).

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Together, these pioneering studies conceptualized the existence of an active protein quality control mechanism. Parallel studies showing that unassembled subunits of the T-cell receptor (TCR) complex rapidly degrades, suggested an ER-based mechanism to dispose of potentially harmful molecules (Lippincott-Schwartz et al. 1988; Bonifacino et al. 1989). Despite these monumental advances, the molecular mechanisms of ERQC remained elusive for years.

Using yeast as a model system for cell biology, investigators taking divergent paths serendipitously converged onto the nascent field of ERQC. Enormous strides were made in these early studies. The Wolf laboratory, focusing on the biogenesis of the vacuole (yeast lysosome), discovered that defective variants of its resident enzymes carboxypeptidase Y and proteinase A (CPY* and PrA*, respectively) never make it to the organelle but instead turn over in a pre-Golgi compartment (Finger et al. 1993). What degrades the misfolded enzymes, however, was unclear. A key genetic interaction discovered by Sommer and Jentsch provided a hint. They found that a *UBC6* loss-of-function mutation suppressed the temperature-sensitive phenotype of *sec61-2*. This suggested that the functionally disruptive mutation in the translocon subunit also causes its degradation (Sommer and Jentsch 1993). More intriguingly, the finding linked the ubiquitin-proteasome system (UPS) to ERQC for the first time. The genetic link was confirmed functionally in follow-up studies and by the discovery that degradation of misfolded cystic fibrosis transconductance regulator (CFTR) protein was blocked by proteasome inhibitors (Ward et al. 1995; Biederer et al. 1996). The Hampton group cemented the generality of the system in ER protein degradation when they showed that the UPS regulates levels of HMG-CoA reductase (Hmg2) according to physiological need (Hampton and Rine 1994; Hampton and Bhakta 1997). Taking the biochemical route, the Brodsky and McCracken groups fractionated yeast cells and established the first in vitro system to study ER protein degradation (McCracken and Brodsky 1996; Werner et al. 1996; Brodsky et al. 1999). It was they who coined the now ubiquitous term, ER-associated degradation or ERAD.

EARLY STEPS OF ERQC: ARREST AND RELEASE

The translocation and folding of secretory and membrane proteins are assisted by ER resident chaperones and their cofactors. These include Hsp70 family members Kar2 and Lhs1, co-chaperones of the DnaJ class (Jem1, Scj1), the nucleotide exchange factor Sil1, the Rot1 membrane-bound chaperone, the lectin-like Cne1, and thiol oxidoreductases (Eps1, Eug1, Mpd1, Mpd2, and Pdi1) (Tachibana and Stevens 1992; Brodsky et al. 1995; Matlack et al. 1997; Plemper et al. 1997; Silberstein et al. 1998; Gillece et al. 1999; Tyson and Stirling 2000; Nishikawa et al. 2001; Zhang et al. 2001; Tsai and Rapoport 2002; Wang and Chang 2003; Heiligenstein et al. 2006; Takeuchi et al. 2008; Hosoda et al. 2009; Sakoh-Nakatogawa et al. 2009; Vembar et al. 2010; Grubb et al. 2012). The fidelity of the selection process is critical because accumulation of aberrant protein conformers is the basis of numerous human diseases. For example, a single point mutation in the gene encoding α 1-antitrypsin causes a severe deficiency resulting in bronchiectasis and pulmonary emphysema. Instead of being properly secreted from cells, the mutant protein is retained in the ER of hepatocytes (Hercz et al. 1978). Familial alleles of the gene encoding CFTR cause their retention and turnover in the ER (Cheng et al. 1990).

How ERQC sorts unfolded from folded proteins is not entirely clear. It could achieve this by actively retaining proteins meant to stay in the ER (unfolded proteins, misfolded proteins, ER resident proteins). All other proteins are simply exported by default. This was the basis of the “bulk flow” paradigm originally proposed by Rothman and coworkers (Wieland et al. 1987). The discovery of ER retention signals and receptors for resident proteins provided the basis of the proposal. The paradigm can be extended to ERQC by putting the job of retention in the hands of ER chaperones, whose very nature it is to associate with unfolded polypeptides. This simple model is confounded by the discovery of a large family of cargo sorting factors, including Erv26 and Erv29, which recognize export signals embedded in folded proteins and

concentrates them at ER export sites (Dancourt and Barlowe 2010). Thus, in principle, an ERQC sorting mechanism could be through the formation of conformational export signals that form only when proteins are properly folded. Molecules failing to form export signals would be retained by default. This mode of sorting, if in operation, is insufficiently comprehensive for ERQC because some misfolded proteins can be exported out of the ER through COPII vesicles (Vashist et al. 2001; Wang and Ng 2010) using export signals that remain functional in the misfolded proteins (Kawaguchi et al. 2010). One purpose of the apparent “leakiness” is to provide a safety valve for conditions of stress, thus allowing other degradative pathways to assist when ERAD is saturated (Spear and Ng 2003). Otherwise, the inability to degrade aberrant proteins can lead to the increase of reactive oxygen species and cell death (Haynes et al. 2004). Taken together, a sorting mechanism that combines both retention and export is likely used by ERQC to provide the needed stringency at steady state and also allow some flexibility to handle increased substrate loads as conditions warrant.

Any sorting mechanism for ERQC is complicated further by the need to distinguish folding intermediates from misfolded proteins. Folding intermediates must stay in the ER because their maturation depends on resident chaperones and modifying enzymes. Misfolded proteins, on the other hand, must be captured and degraded. Some misfolded proteins form insoluble aggregates that are cleared by autophagy (Kruse et al. 2006; Ishida and Nagata 2009; Ishida et al. 2009). How these aggregates are recognized is unclear. Other ER-retained misfolded proteins remain soluble through their chaperone interactions (Nishikawa et al. 2001; Kabani et al. 2003). In some cases, these proteins can traffic out of the ER before they are retrieved from the Golgi for ERAD (Vashist et al. 2001; Kincaid and Cooper 2007; Hirayama et al. 2010). How cells differentiate this class from folding intermediates has puzzled the field for years. Unlike misfolded aggregates, there was no obvious physical difference between the two forms. How does a cell decide that a protein is misfolded and should be degraded versus one that is just in the process of folding?

JUDGE AND JURY: E3 UBIQUITIN LIGASES ORGANIZE SITES OF ERAD SUBSTRATE PROCESSING

In budding yeast, the efforts of numerous groups contributed to the characterization of two ERAD complexes embedded in the ER membrane that function to recognize, translocate, and ubiquitinate substrates for degradation. These can be described as the Hrd1 and Doa10 complexes, named for the E3 ubiquitin ligases that differentiate them (Fig. 1). Hrd1 (also known as Der3) was identified from mutants unable to degrade Hmg2 and CPY* (Hampton

Figure 1. Schematic representation of Hrd1 and Doa10 complexes. Individual subunits of each complex are depicted with their known partners. (Top) The Doa10 complex monitors the folding state of cytosolic domains of membrane proteins (ERAD-C). (Bottom) The Hrd1 complex recognizes lesions of luminal domains of membrane and soluble proteins (ERAD-L) and of lesions within transmembrane domains (ERAD-M).

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et al. 1996; Hiller et al. 1996; Knop et al. 1996a; Hampton and Bhakta 1997; Bordallo et al. 1998; Cronin et al. 2000; Bays et al. 2001b). Doa10 was discovered from a genetic screen seeking genes required for the turnover of proteins bearing the *Deg1* degradation signal (Swanson et al. 2001). Doa10 is dispensable for CPY* degradation, which is soluble, but required for the degradation of some normal and aberrant ER membrane proteins (Swanson et al. 2001; Hoyer et al. 2004; Vashist and Ng 2004). A variety of other factors were identified for ERAD including Cue1, Ubc7, Ubc6, Der1, Hrd3, Cdc48, Npl4, Ufd1, Yos9, Htm1/Mnl1, and Ubx2 (Sommer and Jentsch 1993; Biederer et al. 1996, 1997; Hiller et al. 1996; Hampton and Bhakta 1997; Bordallo et al. 1998; Hill and Cooper 2000; Wilhovsky et al. 2000; Bays et al. 2001b; Hitchcock et al. 2001; Jakob et al. 2001; Nakatsukasa et al. 2001; Rape et al. 2001; Swanson et al. 2001; Walter et al. 2001; Ye et al. 2001; Jarosch et al. 2002; Rabino- vich et al. 2002; Buschhorn et al. 2004; Bhami- dipati et al. 2005; Kim et al. 2005; Neuber et al. 2005; Schubert and Buchberger 2005). Bio- chemical analyses showed that Cdc48 forms a complex with Npl4 and Ufd1, Hrd3 with Hrd1, and Cue1 with Ubc7 (Biederer et al. 1997; Plem- per et al. 1999; Gardner et al. 2000; Hoppe et al. 2000; Meyer et al. 2000). Genetic analysis showed that some genes including *DER1*, *YOS9*, *HTM1/ MNL1*, *HRD1*, and *DOA10* were needed for only subsets of substrates, whereas other genes like *CUE1*, *UBC7*, and *CDC48* were more broadly required (Bordallo et al. 1998; Plemper et al. 1998; Swanson et al. 2001; Walter et al. 2001; Zhang et al. 2001; Taxis et al. 2003; Hoyer et al. 2004; Ravid et al. 2006). A pattern emerged that some genes are specific for substrates contain- ing luminal lesions (ERAD-L), for cytosolic le- sions (ERAD-C), or required for all substrates (Taxis et al. 2003; Hoyer et al. 2004; Vashist and Ng 2004; Willer et al. 2008). The significance of the pattern became clear through direct bio- chemical characterization of ERAD complexes.

Using tandem affinity purification (TAP), Carvalho and Rapoport showed the existence of two distinct complexes (Carvalho et al. 2006). The surprise was not in the differences but in their similarities. Doa10 forms a complex with

Cue1-Ubc7 and with Cdc48-Npl4-Ufd1-Ubx2 (Fig. 1, top). Hrd1 is associated with the same factors plus Hrd3, but also with Der1, Yos9, and a new factor Usa1 (Fig. 1, bottom). Hrd3 con- tains a large amino-terminal luminal domain and a carboxy-terminal transmembrane do- main that interacts with Hrd1. The multispann- ing membrane protein Der1 might participate in the transfer of misfolded proteins from the ER to the cytosol (Knop et al. 1996a). The der- lin-like protein Dfm1 is proposed to be involved in ERAD-C and -L degradation by interacting with Cdc48 (Schubert and Buchberger 2005; Sato and Hampton 2006; Goder et al. 2008; Stolz et al. 2010). Usa1p links Der1 to Hrd1 (Carvalho et al. 2006). Usa1 contains two large amino- and carboxy-terminal cytosolic do- mains joined by two transmembrane segments. Both domains are important for ERAD activity and its interaction with the Hrd1p complex (Kim et al. 2009; Carroll and Hampton 2010). The N and C domains induce oligomerization of the Hrd1 complex and binds Der1, respec- tively (Horn et al. 2009). In this way, Usa1 func- tions as the major structural scaffold for the Hrd1 complex.

The organization of individual compo- nents of the Hrd1 and Doa10 ERAD complexes helped explain the genetic patterns observed previously for substrate specificity. An extended analysis showed that the Hrd1 complex is also required for membrane protein substrates bear- ing lesions within transmembrane segments (ERAD-M) (Carvalho et al. 2006). These ad- vances provided a clear structural framework that was used to guide future studies.

THE VERDICT: SUBSTRATE SELECTION FOR DEGRADATION

Misfolded proteins can expose hydrophobic patches that would be buried in the native state. In the ER lumen, the molecular chaperones Kar2, Scj1, Jem1, and Pdi1 bind hydrophobic peptides and are required for ERAD (Silberstein et al. 1998; Gillece et al. 1999; Nishikawa et al. 2001; Thibault et al. 2011; Grubb et al. 2012). On the cytosolic side, Hsp26, Hsp42, Hsp70, Ydj1, and Hlj1 participate in ERAD depending on the

substrate (Huyer et al. 2004; Youker et al. 2004; Ahner et al. 2007; Park et al. 2007; Vembar et al. 2009). Because most chaperones dissociate from proteins after folding, a prolonged interaction might seem to provide a simple mechanism for substrate selection. It is insufficient, however, because several misfolded proteins are known to accumulate stably in the ER (Knop et al. 1996b; Loayza et al. 1998; Kostova and Wolf 2005; Spear and Ng 2005; Kruse et al. 2006). For this reason, additional biochemical signatures or signals were proposed to be necessary for ERAD recognition.

The best-characterized ERAD determinant exploits the structure of an *N*-linked glycan (Knop et al. 1996b). When the consensus sequence Asn-X-Ser/Thr (in which X is any amino acid other than proline) is encountered by oligosaccharyl transferase, the glycan is transferred *en bloc* from the dolichyl oligosaccharide Glc₃-Man₉-GlcNAc₂-p-p-Dol substrate to the asparagine side chain (Fig. 2) (Burda and Aebl 1999). During folding cycle, glucosidase I and II (Gls1 and Gls2) sequentially removes three glucose residues leaving Man₉-GlcNAc₂. ER mannosidase I (Mns1) next trims the most distal mannose of branch B to generate Man₈-GlcNAc₂. Inhibition of any step impairs glycoprotein ERAD (Jakob et al. 1998; Hitt and Wolf

2004; Clerc et al. 2009). Together, these processing steps are not rapid so they provide a time window for the glycoprotein to fold. Should it remain unfolded, the Htm1 (also called Mnl1) mannosidase complexed with protein disulfide isomerase (PDI), mediates the next crucial step (Clerc et al. 2009; Sakoh-Nakatogawa et al. 2009). In vitro reconstitution experiments showed that the enzyme complex prefers unfolded polypeptides bearing the Man₈-GlcNAc₂ glycan. For these proteins, Htm1-PDI specifically cleaves the terminal mannose residue from the C branch (Fig. 2) (Gauss et al. 2011). This exposes a terminal α 1,6-linked mannose, which is the ligand for the ERAD substrate receptor Yos9 (Buschhorn et al. 2004; Bhamidipati et al. 2005; Kim et al. 2005; Szathmary et al. 2005; Quan et al. 2008).

The Man₇-GlcNAc₂ glycan structure alone is insufficient to target a substrate to ERAD (Xie et al. 2009). It was recognized that only specifically positioned substrate glycans can signal ERAD (Kostova and Wolf 2005; Spear and Ng 2005). It was later shown that the signal is bipartite, consisting of the Man₇-GlcNAc₂ glycan attached to a disordered segment (Xie et al. 2009). In this way, the positioned glycan functions as an intrinsic sensor for protein folding. If the protein folds by the time the signal glycan is processed to

Figure 2. Targeting glycoproteins for degradation. The core GlcNAc₂-Man₉-Glc₃ glycan is rapidly added to the side-chain nitrogen of Asn (N) residues part of the consensus sequence Asn-X-Ser/Thr (N-X-S/T). This posttranslational modification occurs as soon as polypeptides enter the endoplasmic reticulum through the translocon. Subsequently, the three glucoses of branch A are trimmed consecutively by the glucosidase I (Gls1) and glucosidase II (Gls2) to generate GlcNAc₂-Man₉. Mannosidase I (Mns1) cleaves the α 1,2-linked mannose of branch B to produce GlcNAc₂-Man₈. At this stage, folded and glycosylated proteins may leave the ER. However, glycosylated proteins failing to fold are recognized by Htm1/Pdi1, which cleaves the α 1,2-linked mannose of branch C to yield the terminal α 1,6-linked mannose residue as the Yos9 ligand (red circle).

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Man₈-GlcNAc₂, it escapes Htm1-PDI processing, leaving it free to exit the ER. Through this “glycan timer” mechanism, nascent polypeptides exceeding their set folding periods are degraded by ERAD.

A second type of ERAD glycan modification is more enigmatic. It was reported by several groups that some substrates are modified by O-mannosylation (Harty et al. 2001; Vashist et al. 2001; Nakatsukasa et al. 2004; Hirayama et al. 2008). During O-mannosylation, single mannose sugars are transferred covalently to serine and threonine residues, whose total numbers and distributions are currently unknown. The reaction is specifically performed by the Pmt1 and Pmt2 protein mannosyltransferases, which form a complex (Strahl-Bolsinger et al. 1999). The modification is required for efficient degradation of substrates, possibly by maintaining them in a more soluble form (Harty et al. 2001; Hirayama et al. 2008). Interestingly, the *PMT1* and *PMT2* genes are targets of the unfolded protein response, consistent with a stress-related function (Travers et al. 2000). How misfolded proteins are specifically targeted for O-mannosylation is unknown. However, a variety of ER factors including the Hrd1 complex, p24 family proteins, PDI, and Ero1 are associated with the Pmt1/Pmt2 complex suggesting possible mechanisms (Goder and Melero 2011). Furthermore, because ERAD is only nominally compromised when substrate O-mannosylation is reduced, a yet to be determined role in ERQC may be awaiting discovery.

The Hrd1 complex also mediates degradation of membrane proteins bearing defects in their transmembrane segments. Early insight into this mechanism was revealed by studies using Hmg2, a normal transmembrane protein. Hmg2 is the rate-limiting enzyme in sterol biosynthesis, whose levels are regulated by lipid signals within the membrane. When lipid levels in the pathway are high, Hmg2 is degraded by the Hrd1 pathway (Hampton et al. 1996; Gardner et al. 1998, 2001; Gardner and Hampton 1999a,b; Federovitch et al. 2008; Garza et al. 2009). Among misfolded proteins, the Hrd1 complex can also recognize aberrations within transmembrane domains (Carvalho et al.

2006). This mode has been termed ERAD-M. Unlike ERAD-L, whose substrates engage luminal components like Kar2 and Htm1-PDI first, it is the Hrd1 protein itself that recognizes transmembrane distortions (Sato et al. 2009). Some components like Htm1-PDI and Yos9 that are critical for ERAD-L are entirely dispensable for ERAD-M even as both pathways converge in the same Hrd1 complex (Kanehara et al. 2010). In the regulation of sterol biosynthesis, Hmg2 seems to have adapted principles of ERAD substrate recognition for its degradation. Hmg2 contains a conserved sterol-sensing domain (SSD) that detects pathway intermediates (Theesfeld et al. 2011). The SSD mediates a conformational change recognized by the Hrd1 complex that leads to Hmg2 ubiquitination and degradation (Shearer and Hampton 2004, 2005).

The Doa10 complex (Fig. 1, top) is mostly dedicated to substrates found on the cytosolic side of the ER membrane even though it can serve as a complement or backup to the Hrd1 complex (Gnann et al. 2004; Vashist and Ng 2004). Interestingly, not only is Doa10 required for the degradation of misfolded membrane proteins, it also serves as the E3 enzyme for degradation of some misfolded cytosolic proteins (Metzger et al. 2008; Lewis and Pelham 2009). For transmembrane proteins, Doa10 recognizes misfolded cytosolic domains (Huyer et al. 2004; Vashist and Ng 2004; Nakatsukasa et al. 2008). An in vitro ERAD system revealed that a substrate precomplex formed with Hsp70/Hsp40 mediates recognition and ubiquitination by Doa10 (Nakatsukasa et al. 2008). Examples of the requirement for cytosolic chaperones for transmembrane protein ERAD is growing and it is becoming clear that like luminal ERAD components, the requirements vary depending on the substrate (Ahner et al. 2007; Hrizo et al. 2007; Buck et al. 2010; Bell et al. 2011; Needham et al. 2011).

DEAD MAN WALKING: SUBSTRATE RETROTRANSLOCATION

Proteins enter the ER through the Sec61 translocon complex (Walter and Johnson 1994). Because the UPS is on the cytosolic/nucleo-

Figure 3. Retrotranslocation and degradation of ERAD substrates. (A) Proteins targeted for degradation by ERAD complex are retrotranslocated through a “retrotranslocon” (Hrd1, Doa10, and Sec61 are proposed to serve this role but only the E3s are shown for simplicity) with the mechanical force provided by the Cdc48-Npl4-Ufd1 AAA-ATPase complex. Polypeptides are subsequently ubiquitinated as depicted. (B) In the cytosol, Png1 removes N-linked glycans from substrates. Ufd2 can lengthen ubiquitin chains for some substrates. Polyubiquitinated species are recognized by Rad23p and passed to the proteasome for degradation.

plasmic side of the ER membrane, misfolded proteins must retrotranslocate from the membrane (Fig. 3A). The mechanism for this event is unclear. In principle, there could exist multiple mechanisms to account for substrate diversity. For soluble substrates, a pore-like conduit seems to be a requirement. For these molecules, this process is likely more complex than import because substrates can be oligomeric, be modified by disulfide bonds, contain N-linked or O-linked glycans, or any combination of these. Given the small size of the Sec61 pore, it would be inadequate for many proteins to use it also as the retrotranslocon (Van den Berg et al. 2004). For transmembrane proteins containing cytosolic domains, a direct “pulling” mechanism could work in the absence of a pore, but the high energy cost might preclude the possibility. Alternatively, passage of transmembrane domains into a pore-like complex as an early step of retrotranslocation is possible. This would simply be the reverse of the forward membrane integration mechanism (Van den Berg et al. 2004; Ismail et al. 2006).

Some steps of substrate retrotranslocation are resolved. Substrate recognition and targeting are usually coupled events of translocation mechanisms. This seems to be also true of ERAD. The ER chaperones Kar2, Jem1, and

Scj1 are not just early engagers but also keep luminal ERAD substrates soluble, a state needed for retrotranslocation (Nishikawa et al. 2001). As discussed above, the Htm1-PDI complex is a key substrate recognition factor. The Htm1 mannosidase reaction generates the targeting signal recognized by Hrd3 and Yos9 proteins of the Hrd1 complex. Despite their seemingly glycan-specialized function, Hrd3 and Yos9 also bind nonglycosylated misfolded proteins and the core Hrd1 complex mediates the glycan-independent ERAD-L pathway (Bhamidipati et al. 2005; Gauss et al. 2006a,b; Kanehara et al. 2010). Thus, recent evidence reveals that Yos9 can improve the degradation efficiency of some nonglycosylated substrates (Benitez et al. 2011; Jaenicke et al. 2011). Furthermore, recent studies show that the Hrd1 and Yos9 play important roles in ER retention of misfolded proteins (Izawa et al. 2012). Along similar lines, PDI extends its functional versatility to ERAD. It is also required to target the nonglycosylated substrate Δ gp α f to the export channel, to chaperone apolipoprotein B for ERAD, and provide redox activity required for CPY* turnover (Gilce et al. 1999; Grubb et al. 2012).

Polyubiquitination is not just a degradation signal for ERAD; it is also a critical step in retrotranslocation. Even in the earliest studies, it was

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observed that mutants defective in ubiquitination accumulate substrates in the ER (Hiller et al. 1996; Biederer et al. 1997). Soluble substrates can begin the export process but regress to the lumen should ubiquitination fail (Elkabetz et al. 2004). For transmembrane proteins, polyubiquitination is required for their extraction from ER membranes in vitro (Nakatsukasa et al. 2008). The polyubiquitin chain likely acts as a recognition signal for the p97 complex (homolog of yeast Cdc48p complex). The AAA⁺ ATPase protein Cdc48 forms a heterotrimeric complex with the cytosolic proteins Ufd1 and Npl4 and is required for ERAD (Meyer et al. 2000; Bays et al. 2001b; Braun et al. 2002; Jarosch et al. 2002; Rabinovich et al. 2002). Ubiquitinated substrates are retrotranslocated to the cytosol by the action of the Cdc48 complex and targeted to the proteasome for degradation (Ye et al. 2001; Braun et al. 2002). The data suggest that p97 provides the mechanical force to move the polypeptide into the cytosol through ATP hydrolysis cycles (Ye et al. 2003). However, some substrates can retrotranslocate independently of the Cdc48 complex. Here, the mechanical force for extraction is likely provided by the proteasome 19S subunit (Russell et al. 1999; Ng et al. 2007).

The Cdc48 complex is anchored to the ER membrane through Ubx2. Ubx2 connects Cdc48 to the Doa10 complex by binding both (Neuber et al. 2005; Schuberth and Buchberger 2005; Wilson et al. 2006). The Ubx-domain protein Ubx4 is required for the ERAD activity of the Cdc48 complex (Alberts et al. 2009). *UBX4* mutants accumulate substrates associated with Cdc48 suggesting that it is required for the final steps of extraction.

The identity of a translocation channel has been elusive. Although there are several candidates, there is no consensus to date. The most likely reason for the difficulty is the existence of multiple channels or multiple components required to assemble the channel(s). The first candidate proposed was the import channel Sec61 itself (Pilon et al. 1997; Plemper et al. 1998, 1999). Some *SEC61* mutants are defective in ERAD at temperatures in which import is little affected (Plemper et al. 1998). So far, no direct

evidence has been obtained for the export of soluble ERAD substrates through Sec61. However, a short-lived membrane substrate can be cross-linked to Sec61 through an in vivo intermolecular disulfide bond (Scott and Schekman 2008). Interestingly, a cryptic glycosylation site in the amino-terminal cytosolic domain becomes glycosylated, suggesting its transient localization there during the retrotranslocation process. However, a recent study shows that glycosylation could be owing to aberrant reengagement with the Sec61 translocon, which is eventually resolved through degradation via the Hrd1 pathway (Rubenstein et al. 2012). The ERAD factor Der1 was a proposed channel component because its mammalian homolog Derlin-1 can be cross-linked to MHC class I proteins in the process of retrotranslocation (Lilley and Ploegh 2004; Ye et al. 2004). However, some doubt to this hypothesis for the yeast system was raised by conditions that make Der1 entirely dispensable in ERAD (see below). In addition, Der1 is entirely dispensable for ERAD-M (Taxis et al. 2003; Carvalho et al. 2006; Kanehara et al. 2010). However, Der1's essential function for ERAD-L and mostly transmembrane organization suggests a supporting, if not direct, role in forming the channel. Perhaps the most intriguing proposal is that misfolded glycoproteins are transported from the ER for degradation via lipid droplets (Ploegh 2007). In mammalian cells, the formation of lipid droplets and ERAD is linked via the protein AUP1. AUP1 is required for ERAD and localizes to both the Hrd1 complex and lipid droplets (Klemm et al. 2011). Despite this interesting link, a yeast strain defective in the formation of lipid droplets is competent for ERAD (Olmann and Kopito 2011).

More recently, the Hrd1 protein itself was proposed to directly retrotranslocate ERAD-L substrates (Carvalho et al. 2010). Remarkably, overexpressing Hrd1p eliminates the requirement of the complex components Hrd3, Usa1, and Der1. Hrd1p oligomerization is required for this activity and it can be cross-linked with substrate at the early stages of retrotranslocation. Taken together, these data support the idea that Hrd1 itself forms all or parts of the

retrotranslocation channel for ERAD-L. However, this role may not extend to ERAD-M because Hrd1 and Doa10 proteins are dispensable for the retrotranslocation of the fusion protein Hmg2-GFP in vitro (Garza et al. 2009).

LAST RITES: SUBSTRATE UBIQUITINATION AND PROTEASOME DEGRADATION

ERAD substrates engaged for degradation are polyubiquitinated and deglycosylated before being degraded into small peptides by the cytosolic proteasome (Fig. 3B). The essential cytosolic E1 ubiquitin-activating enzyme (McGrath et al. 1991) Uba1 activates ubiquitin by adenylation and attaches ubiquitin to one of its cysteine residues. Activated ubiquitin is subsequently transferred to the ERAD E2 ubiquitin-conjugating enzymes Ubc6 and Ubc7. Finally, ubiquitin is transferred to an ERAD substrate by the action of E3 ubiquitin ligase Hrd1 or Doa10. Both contain an amino-terminal cytosolic RING-type zinc finger domain required for its ligase activity (Bordallo and Wolf 1999). The Hrd1 RING domain binds and accepts ubiquitin from the membrane-associated protein E2 ubiquitin-conjugating ligase Ubc7 (Bays et al. 2001a). Hrd1 was proposed to support self-ubiquitination through its RING domain (Bazirgan et al. 2006). Lysine residues of ERAD substrates are the preferred ubiquitin acceptor but cysteine, threonine, serine, and even the amino terminus can be ubiquitin acceptors (Kerscher et al. 2006). ERAD substrates are polyubiquitinated by their E3s and through the action of the E4 chain-extension enzyme Ufd2 (Nakatsukasa et al. 2008).

Also in the cytosol, N-linked glycans are removed from ERAD substrates before degradation. The cytosolic enzyme Png1 catalyzes this reaction (Suzuki et al. 2000). Cells lacking *PNG1* degrades CPY* nominally slower than wild type but other molecules like the ricin A chain are strongly impaired (Kim et al. 2006). Png1 generates free oligosaccharides (fOS) in the cytosol, which can be analyzed to determine the structures of endogenous ERAD substrate glycans. Analysis of fOS dependent on Png1 revealed the expected Man₇-GlcNAc₂ structure generated by the glycan timer cascade. More sur-

prisingly, fOS structures consistent with Golgi processing were found abundantly, indicating that some ERAD substrates are retrieved from the Golgi before ERAD (Hirayama et al. 2010). Under stress when glucose is limiting, the levels of Png1-generated fOS increases dramatically suggesting elevated ERAD activity under those conditions (Chantret et al. 2011). The amino terminus of Png1 binds Rad23 (Biswas et al. 2004; Wang et al. 2009). Rad23 binds ubiquitinated proteins and transfers them to the proteasome for degradation (Schauber et al. 1998). Rad23 can also interact with Ufd2 (Kim et al. 2004). Interestingly, Ufd2 also binds Cdc48, potentially linking the ERAD machinery to the proteasome under some circumstances (Baek et al. 2011). Together, these findings suggest that ERAD substrates are rapidly polyubiquitinated, deglycosylated, and degraded in a localized environment near the Hrd1 and Doa10 complexes.

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